Supplemental Data

Two MscS Homologs Provide Mechanosensitive Channel Activities in the *Arabidopsis* Root

Elizabeth S. Haswell, Rémi Peyronnet, Hélène Barbier-Brygoo, Elliot M. Meyerowitz, and Jean-Marie Frachisse

Supplemental Experimental Procedures

Resources

Provided below are the locus identifier for each listed gene and, in parentheses, the number for the publicly available mutant line and the formal genetic name for the mutant line used in the text.

MSL4: At1g53470 (SALK_142497, *msl4-1*)
MSL5: At3g14810 (SALK_127784, *msl5-2*)
MSL6: At1g78610 (SALK_06711, *msl6-1*)
MSL9: At5g19520 (SALK_114626, *msl9-1*)
MSL10: At5g12080 (SALK_076254, *msl10-1*)

Microscopy

For confocal imaging, roots from 2-week-old seedlings or leaves from 4-week-old soil-grown plants were mounted in water. For cell-wall counter-staining, tissue was incubated briefly in a 50 μg/ml solution of propidium iodide (Sigma P4170) and rinsed in water before mounting and imaging took place. Excitation was at 488 nm, and emissions were collected with a 505–530 nm BP filter (GFP) or a 590–585 nm BP filter (propidium iodide). A Zeiss LSM 510 laser module was used and images captured with LSM 510 Meta software from Zeiss.

Electrophysiology

Patch-clamp experiments were performed as described [S1] at room temperature with a patch-clamp amplifier (model 200A, Axon Instruments, Foster City, CA) and a Digidata 1322A interface (Axon Instruments). Currents were filtered at 1 kHz, digitized at 3 kHz, and analyzed with pCLAMP8.1 and ORIGIN8.0 software. During patch-clamp recordings, pressure was applied with a syringe, assessed by a pressure monitor (World Precision Instruments, PM01R). In order to statistically compare each peak value given by the Gaussian fit, we calculated the standard error (SE) from the width H of the fit. $H = 2\sqrt{(2\ln(2))}\sigma \approx 2.3548\sigma$, hence $SE = (H/2.3548)/\sqrt{n}$ ($n > 30$), where H = full width at half maximum of the fit curve and σ = standard deviation. During measurements, freshly isolated protoplasts from *Arabidopsis* roots were maintained in bathing medium: 50 mM $CaCl_2$, 5 mM $MgCl_2$, 10 mM MES-Tris, and 0,5 mM $LaCl_3$ (pH 5.6). The pipettes were filled with 150 mM KCl, 2 mM $MgCl_2$, 5 mM EGTA, 4.2 mM $CaCl_2$, and 10 mM Tris-HEPES (pH 7.2), supplemented with 5 mM MgATP. Using a vapor pressure osmometer (model 5500, Wescor), we adjusted the osmolarity with mannitol to 450 mosmol for the bath solution and 470 mosmol for the pipette solution. Gigaohm resistance seals between pipettes (pipette resistance, 0.8–2 MΩ) coated with Sylgard (General Electric) pulled from capillaries (Kimax-51, Kimble Glass) and protoplast membranes were obtained with gentle suction leading to the whole-cell configuration. We monitored the quality of the seal throughout each experiment by periodically checking the whole-cell capacitance and making sure that the access resistance remained below 1.5 MΩ. The excision of the patch, leading to the outside-out configuration, was used in some experiments. The liquid junction potentials were measured according to the method of Neher [S2], and we have corrected all potentials given by subtracting the junction potentials. The membrane tension was calculated according to Laplace's law for a thin-walled sphere equilibrium, $T = Pd/4$, where T = tension on the membrane, P = applied pressure, and d = cell diameter. The protoplast diameter was deduced from cell capacity where 1 pF was assumed to equal 100 μm^2.

Plant Growth and Genetics

Arabidopsis thaliana plants were grown on soil or on 4.3 g/liter Murashige and Skoog salts supplemented with 3% sucrose and 8 g/liter phytagar under continuous light. Insertional mutants in the Columbia ecotype were obtained from the SALK Institute, and homozygous lines were identified by PCR genotyping. Primer pairs 19520.F3/19520.R5 and 12080.F1/12080.R1 were used for amplifying the wild-type alleles of *MSL9* and *MSL10*, respectively, and primer pairs LBa/19520.R5 and LBa/12080.R1 were used for amplifying the insertion alleles. PCR products spanning the 3′ insertion junction of *msl9-1* and *msl10-1* were cloned into pGEM T-Easy according to the manufacturer's instructions, then sequenced. Double-mutant lines were generated by standard crossing protocols, and double mutants were identified in the F2 generation by PCR genotyping as described above. Transgenic constructs were introduced into plants by *Agrobacterium*-mediated transformation by the standard floral-dip method [S3].

In Vitro Plant Growth Conditions for Patch-Clamp Experiments

Seedlings were grown, in vitro, in a Versatile Environmental Test Chamber from Sanyo Electric Biomedical Co. at 21°C with a 16 hr day length on media containing 5 mM KNO_3, 2.5 mM K_2HPO_4/KH_2PO_4 (pH 6.0), 2 mM $MgSO_4$, 1 mM $Ca(NO_3)_2$, 1 mM MES, 50 μM Fe-EDTA, Murashige and Skoog microelements [S4], 10 g/liter sucrose, and 7 g/liter agar.

Histology

For detection of β-glucuronidase (GUS) activity, tissue was fixed for 30 min in ice-cold 90% acetone, then incubated overnight at 37°C in 0.5 μg/ml 5-bromo-4-chloro-3-indolyl β-D-glucuronic acid, 100 mM $NaPO_4$ (pH 7), 0.1% Triton X-100, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, and 10 mM EDTA. Samples were then dehydrated through an ethanol series and photographed. For plastic embedding, tissue stained as above for GUS was fixed in cold 1× PBS, 4% paraformaldehyde, 1% glutaraldehyde, and 0.1% Tween-20 overnight, then dehydrated through an ethanol series. Infiltration and polymerization with JB-4 Plus (Electron Microscopy Sciences) was performed according the manufacturer's recommendations. Sections of 3–4 μm were made with a glass blade, then dried onto glass slides and photographed.

Cloning and Subcloning

Plasmid constructs were made with Gateway technology (Invitrogen). PCR products generated as described below with Pfu polymerase (Stratagene) were cloned into pENTR/D-TOPO (Invitrogen) to create entry vectors, then recombined into the appropriate destination vectors, which were provided by the Plant Systems Biology Department, Ghent University, Belgium [S5]. For creation of pENTR-MSL9p and pENTR-MSL10p, the predicted promoter sequences (as identified by the *Arabidopsis Cis*-Regulatory Element Database [S6]) were amplified from genomic DNA with primer sets 19520p.F1/19520p.R1 and 12080p.F1/12080p.R1, respectively. These pENTR constructs were then used in recombination reactions with pBGWFS7 for creating GFPGUS reporter transgenes. For creation of 35Sp::MSL9 and 35Sp::MSL10, MSL9 (U23473) and MSL10 (U21634) cDNAs were obtained from the ABRC. PCR fragments were generated with the primer pairs 19520.F1B/19520.R1 and 12080.F1B/12080.R2 and cloned into pENTR/D-TOPO for creating pENTR-MSL9 and pENTR-MSL10. Similarly, for creation of 35Sp::MSL9∼GFP and 35Sp::MSL10∼GFP, PCR fragments were generated with the primer pairs 19520.F1B/19520.R2 and 12080.F1B/12080.R3 and cloned into pENTR/D-TOPO to create pENTR-MSL9f and pENTR-MSL10f. These entry vectors were then used in recombination reactions with pB2GW7 for creating overexpression constructs or with pK7FWG2 for creating C-terminal GFP fusions, both under the control of the 35S promoter. Subsequently, for creating MSL9p::MSL9∼GFP and MSL10p::MSL10∼GFP, promoters and GFP fusion coding sequences from the Gateway destination vectors described above were subcloned into the plasmid BJ36 containing the 3′ region from octopine synthase (3′ OCS). The *MSL9* promoter was amplified from pENTR-MSL9p, and the *MSL10* promoter was amplified from pENTR-MSL10p, and these were cloned into the SalI and BamHI sites of BJ36-3′ OCS. MSL9∼GFP and MSL10∼GFP were amplified from pK7FWG2-MSL9f or pK7FWG2-MSL10f and added at the BamHI site of BJ36. After sequencing, Not I fragments containing promoters, open reading frames, and the 3′ OCS were released from BJ36 vectors and inserted into the unique Not I site in the binary vector pBART for plant transformation.

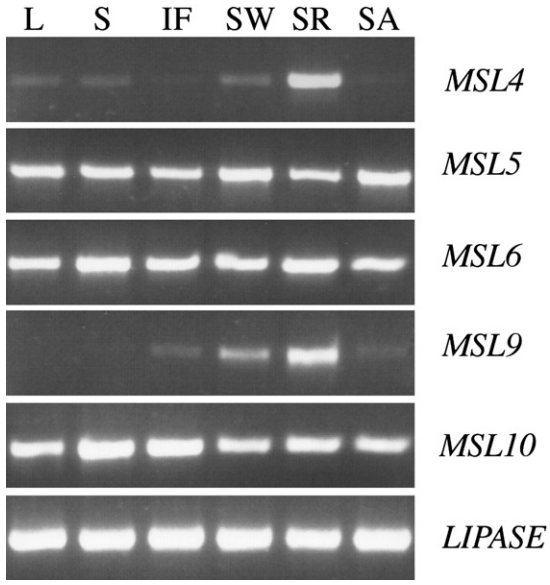

Figure S1. Tissue-Specific Expression of *MSL* Genes

An agarose gel showing RT-PCR products from leaf (L), stem (S), inflorescence (IF), whole seedling (SW), seedling root (SR), and aerial portions of the seedling (SA). Gene-specific primers from *MLS4*, *MSL5*, *MSL6*, *MSL9*, and *MSL10* as described in Table S1 were used. *LIPASE* expression indicates that equal amounts of cDNA template were used in each reaction.

RT-PCR

RNA was purified from 100 mg of tissue collected from the shoots and roots of 10-day-old seedlings grown on vertical agar plates, and from various tissues of 3-week-old soil-grown plants, with the QiaQuick RNA preparation kit (QIAGEN), including treatment with DNase for 30 min at 37°C. We used 2 μg of this RNA as a template for first-strand cDNA synthesis by mixing it with 0.5 μg oligo(dT) (Promega), 1× first-strand buffer, 10 mM DTT, 2 mM dNTPs, and Superscript Reverse Transcriptase (Invitrogen) and then incubating it overnight at 37°C. PCR was performed with HotStar Taq polymerase (QIAGEN) in 25 μl reactions for 25 cycles with 1 μl cDNA template. In Figure 2B, LIP.F/LIP.R, 9.F4/9.R4, and 12080.F6/12080.R2 were used. In Figure S1, the following primer sets were used: LIP.F/LIP.R, MSL9.F4/MSL9.R4, MSL10.F1/MSL10.R1, MSL4.F7/MSL4.R2, MSL5.F1/MSL5.R4, and MSL6.F1/MSl6.R5. Aliquots (10 μl) were run on a 1.5% agarose gel and visualized with ethidium bromide under UV light. Primer sequences are listed in Table S1.

Protoplast Isolation and Transient Transformation

Roots were cut into 1–2 mm segments without the tips and digested for 30 min at 30°C under hyperosmotic conditions (2 mM $CaCl_2$, 2 mM $MgCl_2$, 1 mM KCl, MES/Tris [pH 5.5], 0.3% cellulase RS [Onozuka RS, Yakult Honsha Co.], 0.03% pectolyase Y23 [Kikkoman Corporation], 0.35% Albumine serum bovin and mannitol to 600 mOsmol). For enzyme removal, the preparation was centrifuged for 5 min at 800 rpm and washed twice with 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM MES (pH 5.5), and mannitol to 450 mOsmol. Filtering the suspension (through a 300 μm nylon mesh) yielded protoplasts. For transformation, protoplasts were mixed with 7 μg of plasmid DNA in a solution containing 25% PEG 6000, 0.45 M mannitol, and 0.1 M $Ca(NO_3)_2$ (pH 9) and incubated in the dark at 24°C for 20 min. Then the protoplasts were washed twice with 0.275 M $Ca(NO_3)_2$ and resuspended in a solution (used for cell-suspension culture) as described in [S7]. Finally, protoplasts were incubated overnight in the dark at 24°C. Protoplasts were cotransformed both with 35Sp::*YFP* in the pMon vector and with *35Sp::MSL9* or *35Sp::MSL10* in the pB2GW7 vector, via a protocol adapted from [S8]. We only used fluorescent protoplasts, indicating a cotransformation.

Phenotypic Analysis of the *msl4-1;msl5-2;msl6-1;msl9-1;ms10-1* Quintuple Mutant

Wild-type and mutant plants were grown on solid MS media adjusted to 50–200 mOsmol with mannitol. Germination, seedling health, and root growth on media containing 0–130 mM mannitol, 0–130 mM sucrose, 0.1–250 mM KCl, and 0.1– 250 mM NaCl were tested, as well as root growth in response to transfer to these media from MS or transfer from these media to MS. Recovery from a 30 min shock in 0.6 M NaCl was also tested. We tested growth on media containing 0.4%–2% agarose, 6%–20% sand, or spherical glass beads (to ~2 mm). We monitored interaction of the root tip with a glass barrier as described in [S9]. We tested dehydration (20 min without water at 24°C) as well as rehydration stresses on plantlets grown under hydroponic conditions, and the growth and survival of submerged, dehydrated, or rehydrated 2-week-old plants grown in soil. During drought, we monitored stress temperature of the leaves with an infrared camera. Reorientation of root growth in response to gravity change was

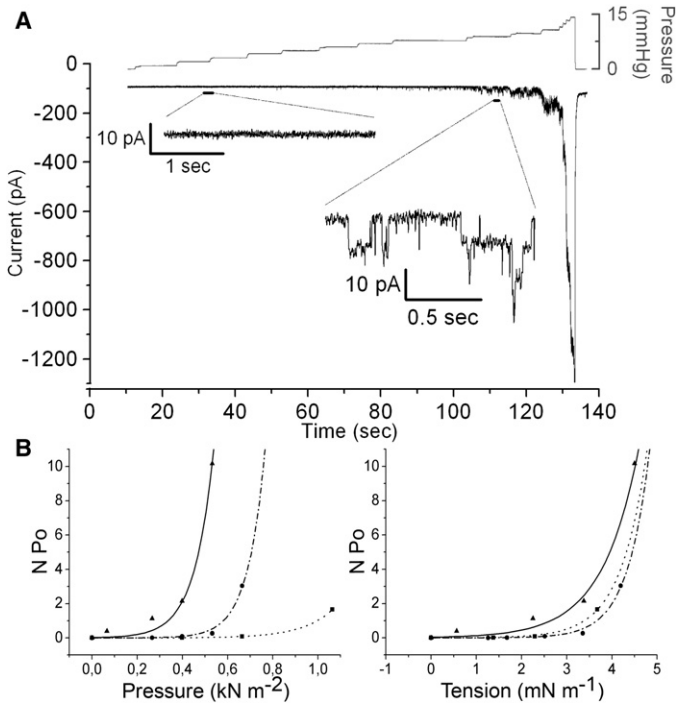

Figure S2. MS Channels Can Be Resolved in Wild-Type Protoplasts via a Whole-Cell Configuration

(A) A representative trace of the wild-type current activation in response to positive pressure, in the whole-cell configuration, at −182 mV. At low positive pressure, a stable and consistent background current of 27 ± 31 pA/pF (n = 38 protoplasts) without unitary channel activity was observed (left inset). When intermediate levels of positive pressure (1–9 mm Hg) were introduced through the patch pipette, the activation of unitary channel activities was observed (right inset). At higher pressures, up to 12 mm Hg, unitary channel activities were no longer resolvable, and a large peak current was elicited; releasing the pressure restored the initial current. We thus chose in this study to analyze events that occur under intermediate levels of pressure, where few channels are activated and each opening and closing event is clear.

(B) The open probability P_o multiplied by the number N of channels in the patch was plotted against applied pressure (left) or tension, calculated from Laplace's law (right) for three independently tested protoplasts (one protoplast per curve). All three curves resemble the early phase of the Boltzmann distribution of the open-probability pressure dependence previously described for mechanosensitive channels in bacteria [S14, S15]. When MS channel open probabilities are plotted against the calculated tension *(right)*, the curves are gathered, indicating that this MS channel seems to be regulated by membrane tension. Ionic conditions were the same as those described in the Supplemental Experimental Procedures.

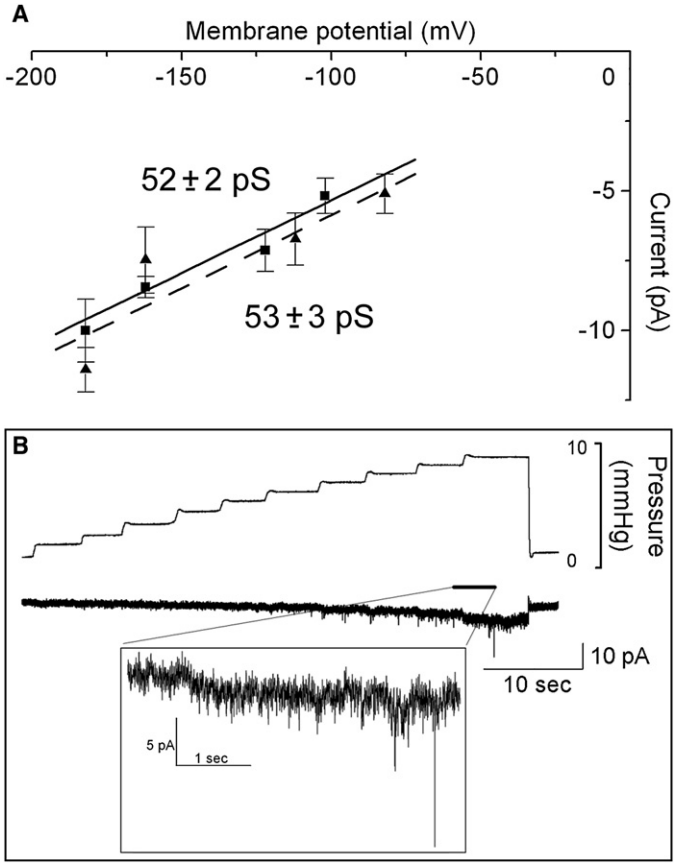

Figure S3. Chloride Permeates Preferentially through the Wild-Type Channel

(A) I/V curve of either wild-type protoplasts (n = 3) under standard bath conditions (5 mM $MgCl_2$ and 50 mM $CaCl_2$, indicated by the continuous line) or the same protoplasts under conditions in which external $CaCl_2$ was replaced with 1 mM $CaCl_2$ and 98 mM TEACl (dashed line). Error bars indicate the standard error of the values obtained from n = 3 protoplasts at each point in the curve.

(B) Replacing the chloride of the internal solution with the large MES anion abolished the wild-type channel activity. The membrane potential was clamped at −182 mV (n = 4).

monitored, and wave assays were performed as described [S10]. The induction of touch- and wound-inducible genes was assayed by semiquantitative RT-PCR, and peroxidase production in response to wounding with forceps was assayed by DAB staining [S11]. Thigmotropic response to repeated touch, wind (provided by a fan), and water spray [S12] was monitored in 2-week-old plants over a 2 week period. Cellular ion concentrations were determined by ionomic analysis [S13]. No differences between wild-type and mutant plants were observed under any of the above conditions.

Supplemental References

S1. Hamill, O.P., Marty, A., Neher, E., Sakmann, B., and Sigworth, F.J. (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. Pflugers Arch. *391*, 85–100.

S2. Neher, E. (1992). Correction for liquid junction potentials in patch clamp experiments. Methods Enzymol. *207*, 123–131.

S3. Clough, S.J., and Bent, A.F. (1998). Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J. *16*, 735–743.

S4. Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco cultures. Physiol. Plant. *15*, 473–497.

S5. Karimi, M., Inze, D., and Depicker, A. (2002). GATEWAY vectors for Agrobacterium-mediated plant transformation. Trends Plant Sci. *7*, 193–195.

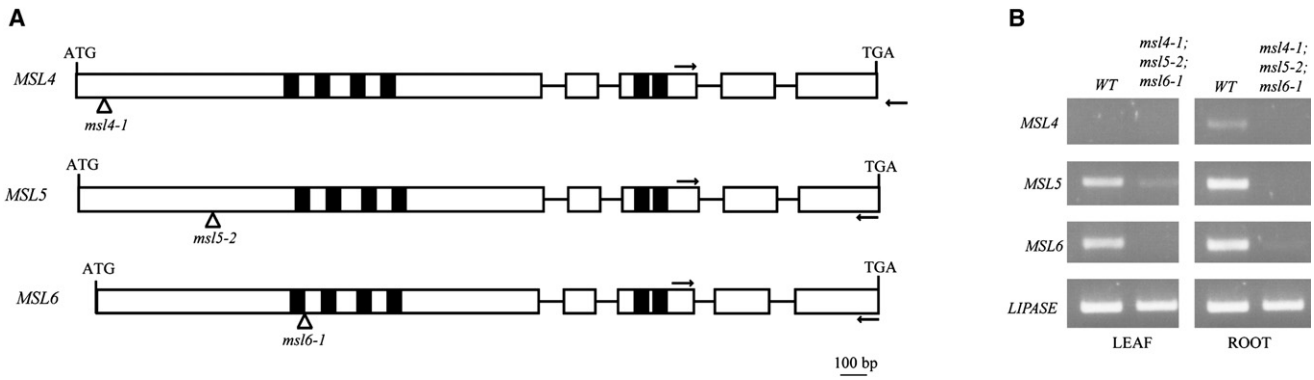

Figure S4. Characterization of the *MSL4, MSL5,* and *MSL6* genes and the *msl4-1;msl5-2;msl6-1* Triple Mutant

(A) Structure of *MSL*4, *MSL5*, and *MSL6* and the location of T-DNA inserts used in this study. White boxes represent exons, and lines represent introns. Black boxes mark the approximate location of TM helices. Arrows indicate the approximate location and direction of the primers used for the RT-PCR in (B). Primer arrows and black boxes indicating TM helices are not to scale.

(B) Ethidium bromide (EtBr)-stained gel showing semiquantitative RT-PCR products from wild-type and *msl4-1;msl5-1;msl6-1* mutant tissue. *MSL4*, *MSL5*, and *MSL6* mRNAs are not detected in the root tissue of mutant plants. A low level of *MSL5* mRNA is still detectable in the leaf.

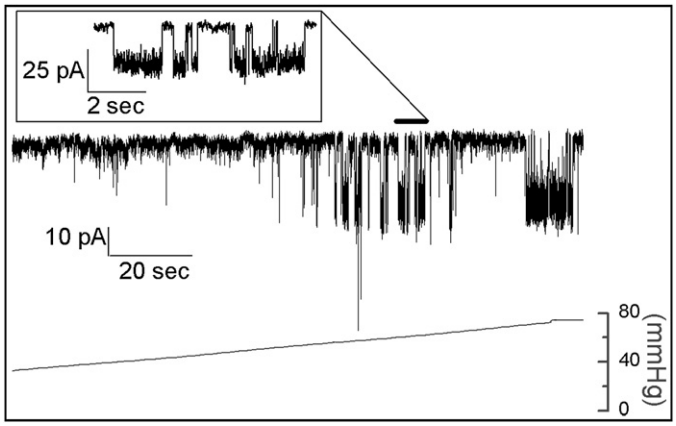

Figure S5. Activation of MSL10 in the Excised Outside-out-Patch Configuration

The channel activation of MSL10 transiently expressed in the double msl9-1;msl10-1 mutant by the use of pressure ramp is shown. The membrane potential was clamped at −182 mV. Ionic conditions are described in the Supplemental Experimental Procedures.

S6. Davuluri, R.V., Sun, H., Palaniswamy, S.K., Matthews, N., Molina, C., Kurtz, M., and Grotewold, E. (2003). AGRIS: Arabidopsis gene regulatory information server, an information resource of Arabidopsis cis-regulatory elements and transcription factors. BMC Bioinformatics *4*, 25.

S7. Droillard, M., Boudsocq, M., Barbier-Brygoo, H., and Lauriere, C. (2002). Different protein kinase families are activated by osmotic stresses in Arabidopsis thaliana cell suspensions. Involvement of the MAP kinases AtMPK3 and AtMPK6. FEBS Lett. *527*, 43–50.

S8. Mathur, J., and Koncz, C. (2006). PEG-mediated protoplast transformation with naked DNA. In Methods in Molecular Biology, Volume 82, J. Martinez-Zapater and J. Salinas, eds. (Totowa, NJ: Humana Press).

S9. Massa, G.D., and Gilroy, S. (2003). Touch modulates gravity sensing to regulate the growth of primary roots of Arabidopsis thaliana. Plant J. *33*, 435–445.

S10. Okada, K., and Shimura, Y. (1990). Reversible root tip rotation in Arabidopsis seedlings induced by obstacle-touching stimulus. Science *250*, 274–276.

S11. Orozco-Cardenas, M., and Ryan, C.A. (1999). Hydrogen peroxide is generated systemically in plant leaves by wounding and systemin via the octadecanoid pathway. Proc. Natl. Acad. Sci. USA *96*, 6553–6557.

S12. Braam, J., and Davis, R.W. (1990). Rain-, wind-, and touch-induced expression of calmodulin and calmodulin-related genes in Arabidopsis. Cell *60*, 357–364.

S13. Salt, D.E., Baxter, I., and Lahner, B. (2008). Ionomics and the study of the plant ionome. Annu. Rev. Plant Biol. *59*, 709–733.

S14. Kloda, A., and Martinac, B. (2001). Molecular identification of a mechanosensitive channel in archaea. Biophys. J. *80*, 229–240.

S15. Berrier, C., Besnard, M., Ajouz, B., Coulombe, A., and Ghazi, A. (1996). Multiple mechanosensitive ion channels from Escherichia coli, activated at different thresholds of applied pressure. J. Membr. Biol. *151*, 175–187.

Table S1. Oligos Used in This Study

Number	Name	Sequence
696	19520.F3	CAAGGGGTTCTTTTGACAGG
784	19520.R5	TCCAAGCAATCAGAACCAGA
623	12080.F1	ATGGCAGAACAAAAGAGTAGTAACG
624	12080.R1	CTTACTGCGCATCTCTCTGTTCAG
500	Lba	TGGTTCACGTAGTGGGCCATCG
726	19520p.F1	CACCATATCCTGAGCTGTGAGACTC
727	19520p.R1	CCCTAAGTCACTTCCAACCAATAAGTTTG
728	12080p.F1	CACCGACGAATTCTTCGTTCGATTG
729	12080p.R1	TCCAATGCTACTACCATCCAATTTCAC
644	19520.F1B	CACCATGGCTGAGAGGAGAGTCAGTAAC
621	19520.R2	TTTGTGACCAGTGAGATTGACATCTTG
660	12080.F1B	CACCATGGCAGAACAAAAGAGTAGTAACG
663	120808.R3	GTTCTTCTTTGTGAGATTAATGTCTTGAGG
620	19520.R1	TCAAAAGTGACCAGTGAGATTGACATC
662	12080.R2	TCAGTTCTTCTTTGTGAGATTAATGTCTTG
512	LIP.F	GTGTGAGAGGTCTCGTTGATTGCC
513	LIP.R	TTCCTGCAACGTTGGAAGATGCTGTC
697	19520.F4	TCTGGTTCTGATTGCTTGGA
706	19520.R4	TTTCCGGTGTCGGCATCTTC
702	12080.F6	GCAACGACTAAGGTTTTGCTG
662	12080.R2	TCAGTTCTTCTTTGTGAGATTAATGTCTTG
746	53470.F7	GCTTCTCATTCTGGGAATCG
674	53470.R2	TCAAACACTGCGTTGTTGATTCC
631	14810.F1	ATGGCGGCTGTCGATTCAAC
741	14810.R4B	CCATTTCCACAAATCCAGCTTCC
633	78610.F1	GAGAGACGGAAGCTACGATTTTTGG
789	78610.R5	GTTCCCAGAGTTTCTTCTTACGCAAG